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(71) Applicant *for all designated States except US*: DSM N.V. [NL/NL]; Het Overloon 1, NL-6411 TE Heerlen (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: VAN DOOREN, Theodorus, Johannes, Godfried, Marie [NL/NL]; Henri Tijssenstraat 56, NL-6042 BZ Roermond (NL). MOODY, Harold, Monro [GB/NL]; Hoogzwanenstraat 148, NL-6211 BZ Maastricht (NL). SMEETS, Johanna, Christina, Maria [NL/NL]; Wilgenroos 1, NL-6002 TV Weert (NL).

(74) Agent: JACOBS, Monique, Sophie, Nicole; Octrooibureau DSM, P.O. Box 9, NL-6160 MA Geleen (NL).

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(54) Title: PROCESS FOR THE PREPARATION OF A β-LACTAM ANTIBIOTIC

(57) Abstract

Process for the preparation of a β-lactam antibiotic in which a β-lactam nucleus is subjected to an enzymatic acylation reaction with the aid of an acylation agent at a molar ratio of acylation agent/β-lactam nucleus of less than 2.5, with the acylation agent and/or the β-lactam nucleus being supersaturated in the reaction mixture during at least part of the acylation reaction. In the process, a concentrated slurry or solution, for instance, of the β-lactam nucleus and/or the acylation agent with a different pH or a higher temperature than the pH or temperature at which the acylation reaction is carried out is added to the reaction mixture during the acylation reaction. Both the β-lactam nucleus and the acylation agent may be supersaturated in the reaction mixture.

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PROCESS FOR THE PREPARATION OF A β-LACTAM ANTIBIOTIC

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The invention relates to a process for the preparation of a β-lactam antibiotic in which a β-lactam nucleus is subjected to an enzymatic acylation reaction with the aid of an acylation agent at a molar
10 ratio of acylation agent/β-lactam nucleus of less than 2.5.

A similar process is disclosed in for example WO-A-96/23897.

The yield of β-lactam antibiotic to be
15 achieved in the enzymatic acylation reaction according to the prior art per amount of β-lactam nucleus employed and per amount of acylation agent employed is in general relatively low inasmuch as the β-lactam nuclei and/or the β-lactam antibiotics often are
20 relatively instable, whilst the reaction time is relatively long because of the usually low solubility of the reactants. Moreover, at the relatively low acylation agent to β-lactam nucleus ratio mentioned, only a relatively low yield of β-lactam antibiotic can
25 be achieved per amount of β-lactam nucleus employed.

The invention provides a process in which in an enzymatic acylation reaction a shorter reaction time is achieved and a higher yield of β-lactam antibiotic is achieved per amount of β-lactam nucleus
30 and/or per amount of acylation agent employed, with the ratio of the amount of acylation agent employed to β-lactam nucleus being relatively low.

This is achieved according to the invention in that the acylation agent and/or the β-lactam nucleus

are/is supersaturated in the reaction mixture during at least part of the acylation reaction.

The applicant has found that it is possible to achieve a high degree of supersaturation of the β-
5   lactam nucleus and/or the acylation agent in the reaction mixture and that, especially surprisingly, the supersaturation can be kept stable for hours. This allows the concentration of the β-lactam nucleus and/or the dissolved acylation agent to be strongly increased,
10   so that the reaction proceeds more rapidly, with less degradation of the β-lactam antibiotic and/or the reactants and also with a higher yield of β-lactam antibiotic per amount of β-lactam nucleus employed and/or per amount of acylation agent employed. This
15   also results in a higher production capacity.

In addition, it is known from literature, for example WO-A-92/01061, that a high yield of β-lactam antibiotic per amount of β-lactam nucleus can be obtained by applying a high molecular ratio between the
20   acylation agent and the β-lactam nucleus. However, a drawback of applying a high molecular ratio between the acylation agent and the β-lactam nucleus is that large amounts of acylation agent are lost as a result of hydrolysis of the acylation agent (and possibly the β-
25   lactam antibiotic). Consequently, a low synthesis/hydrolysis ratio (S/H), the molar ratio between synthesis product (β-lactam antibiotic) and hydrolysis product, is realized. Moreover, it has been found that working up the β-lactam antibiotic is often
30   hampered by a relatively large amount of hydrolyzed acylation agent relative to β-lactam antibiotic being

present in the reaction mixture obtained after the enzymatic acylation reaction, as a result of which a smaller amount of β-lactam antibiotic can be isolated.

For the purposes of the present invention, the yield of β-lactam antibiotic per amount of reactant (β-lactam nucleus or acylation agent) to be achieved in the acylation reaction means the (molar) amount of β-lactam antibiotic formed in the acylation reaction per (molar) amount of reactant employed.

For the purposes of the present invention, the solubility of a compound in a mixture means the dissolved concentration of the compound in the presence of all other components of the mixture, expressed in mmol/litre or mass%. The solubility is measured by dissolving the compound at constant pH and temperature and in the presence of all components of mixture. Thereafter, the solubility can be calculated from the amount of the compound dissolved on reaching equilibrium (saturated solution).

For the purposes of the present invention, a compound is supersaturated in a mixture when the dissolved concentration of that compound in the mixture is greater than the solubility. The supersaturation factor means the ratio between the two aforementioned solubilities (supersaturated divided by saturated). The supersaturation factor to be achieved and the time during which supersaturation is maintained depend on a number of factors such as the nature and concentration of the compound, the nature and concentrations of the other components in the mixture, the pH and the temperature. The supersaturation factor to be obtaiend depends largely on the compound involved and is

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preferably larger than 2, more particularly larger than
5.

The concentration of the dissolved β-lactam
nucleus is expressed as the amount of dissolved β-
5   lactam nucleus in moles per kg of liquid reaction
mixture; the total concentration of dissolved and
undissolved β-lactam nucleus is expressed as the amount
of β-lactam nucleus in moles per kg of the total
reaction mixture; the total reaction mixture may
10   contain, besides the solution, a plurality of solids,
for example β-lactam nucleus, β-lactam antibiotic,
(hydrolyzed) acylation agent and immobilized enzyme.
Similar definitions are applicable for the acylation
agent and the β-lactam antibiotic.

15   A mixture in which the β-lactam nucleus or
the acylation agent, respectively, is supersaturated
can be obtained by means of for example a pH shift. To
that end, if necessary, a concentrated mixture can
first be prepared as a slurry or a solution by
20   dissolving β-lactam nucleus or acylation agent,
respectively, present in solid form with the aid of for
example a pH increase or a pH decrease, or a pH
decrease, respectively. It is preferred for the β-
lactam nucleus and/or the acylation agent to be
25   dissolved in the mixture obtained. However, it is also
possible for a portion of the β-lactam nucleus and/or
the acylation agent to be still present in solid form.
Subsequently, this slurry or solution can be subjected
to a pH decrease or a pH increase, or a pH increase,
30   respectively. In this way, a slurry or solution is
obtained in which the β-lactam nucleus or acylation

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agent is supersaturated.

Any solid β-lactam nucleus present can be dissolved by for example decreasing the pH until a pH lower than 3, preferably lower than 2, in particular

5   lower than 1 is reached; or by increasing the pH to a pH higher than 6, preferably higher than 7, in particular higher than 8. In practice, the final pH is preferably chosen such that the β-lactam nucleus goes only just completely into solution so that as

10   concentrated a solution as possible is obtained. In practice, the concentrated solution will usually have a concentration of the β-lactam nucleus of at least 5 wt.%. The final pH will usually be lower than 10 and greater than 0.

15   Subsequently, a supersaturated solution can be obtained from a solution, whose pH may or may not have been decreased or increased, by increasing or decreasing the pH to a value between for example 3.0 and 9.0, preferably between 4.0 and 8.5, in particular

20   between 4.5 and 8.0.

Any solid acylation agent present can be dissolved by for example decreasing the pH until a value lower than 8.0, preferably lower than 6.5, in particular lower than 5.0 is reached; preferably, the

25   final pH is chosen such that the acylation agent is only just completely dissolved and thus as concentrated a solution as possible is obtained. In practice, the concentrated solution will have a concentration of the acylating agent of at least 5 wt.%. The final pH will

30   usually be greater than 1.

A mixture supersaturated with for example the acylation agent can be obtained from a mixture,

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preferably a solution, whose pH may optionally have been reduced, by increasing the pH to a value greater than for example 4.5, preferably greater than 5.5, in particular greater than 6.0.

Another manner of obtaining a mixture in which the β-lactam nucleus and/or the acylation agent are/is supersaturated is for example by a temperature decrease, optionally after any solid β-lactam nucleus and/or solid acylation agent present have/has first at least partly been dissolved by a temperature increase or pH change.

Dissolution can be effected by for example a temperature increase until for example (virtually) all solid matter is in solution, for example to a temperature higher than 15°C, preferably higher than 20°C, in particular higher than 25°C. Subsequently, a supersaturated slurry or solution can be obtained from the obtained mixture by decreasing the temperature to a temperature lower than 20°C, preferably lower than 15°C, in particular lower than 10°C.

The supersaturated mixture preferably is prepared by changing the pH inasmuch as a higher supersaturation factor can then be achieved. In practice, a pH change and a temperature decrease will usually be applied simultaneously in the preparation of a supersaturated solution.

In a suitable embodiment of the process according to the invention first a mixture in which the β-lactam nucleus and/or the acylation agent are/is supersaturated is prepared, whereupon the acylation reaction is started by for example adding (immobilized) enzyme. The concentration of the reactants will

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decrease during the acylation reaction so that
supersaturation will have gone by the end of the
acylation reaction. In another suitable embodiment the
acylation reaction is started with a portion of the β-
5 lactam nucleus and/or the acylation agent, which may or
may not be supersaturated, whereupon supersaturation is
either maintained or brought about by adding to the
reaction mixture, for example by titration, a
concentrated mixture of the β-lactam nucleus and/or the
10 acylation agent with a different pH or a higher
temperature than the pH or temperature at which the
acylation reaction is carried out. In practice, the β-
lactam nucleus and the acylation agent may both be
present in supersaturated condition during the
15 acylation reaction, for example by metering into the
acylation reactor a concentrated solution or slurry of
the β-lactam nucleus (with a high pH or optionally with
a low pH) and at the same time a concentrated solution
or slurry of the acylation agent (with a low pH).
20 In so doing, the pH can if desired be kept constant
during the acylation reaction by for example titration.
        The process according to the invention can
very suitably be used in the preparation of cefaclor.
Cefaclor exhibits poor stability at high pH (> 6.5)
25 whilst the solubility of the corresponding β-lactam
nucleus (7-amino-3-chloro-cef-3-em-4-carboxylic acid;
7-ACCA) is low at those pH values (about 6.0-6.5) at
which degradation of cefaclor still is relatively low.
As a result, the yield of cefaclor was low at both
30 relatively high and relatively low pH, so that a
technically/commercially attractive process was not
possible. The yield of cefaclor per amount of 7-ACCA

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employed and per amount of acylation agent employed
could admittedly be improved by using for example
naphthol as a complexing agent; the use of such toxic
auxiliary materials in the preparation of antibiotics
5   is, however, disadvantageous in that they need to be
completely removed, which entails additional process
steps, with a strongly negative effect on process
economics. Surprisingly, it has been found that the
process according to the invention allows an
10   exceptionally high supersaturation factor (> 10) to be
achieved so that the acylation reaction can be carried
out at a relatively low pH (6.0-6.5) whilst, yet, the
concentration of the β-lactam nucleus is sufficiently
high. Thus, it is now possible to prepare cefaclor in
15   high yields through enzymatic acylation without using
auxiliary materials so that a technically/commercially
attractive process can be realized.
          Another application of the process
according to the invention lies in the preparation of
20   for example ampicillin by acylation of 6-
aminopenicillanic acid (6-APA) with the aid of D-phenyl
glycine amide (FGA). Since 6-APA and ampicillin degrade
relatively quickly at high concentration and at high pH
it is important to have the acylation reaction proceed
25   as rapidly as possible at as low a pH as possible. By
for example increasing the pH of a mixture of 6-APA and
FGA to a value between 7.0 and 8.0 and then immediately
lowering it to a pH between 6.0 and 6.5 and then
immediately initiating the enzymatic reaction it has
30   been found possible to achieve, in a short period of
time, a high yield of ampicillin per amount of 6-APA
employed and per amount of acylation agent employed,

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with a relatively high concentration of 6-APA being
present in the solution only very briefly and with
degradation of ampicillin being kept low at the
relatively low pH value. At the same time, hydrolysis
5   of FGA to D-phenyl glycine (FG) is restricted.

The process according to the invention can
also be applied with advantage in the enzymatic
preparation of cephalexin through acylation of 7-
aminodesacetoxycephalosporanic acid (7-ADCA) with the
10   aid of D-phenyl glycine amide (FGA). The acylation
reaction is usually carried out at a relatively high pH
value, for example between 7.5 and 8.5, and is attended
by (unwanted) hydrolysis of FGA into D-phenyl glycine
(FG). The reaction can be accelerated, and hydrolysis
15   of the acylation agent can be limited, by applying the
process according to the invention, for example by
first raising the pH of a mixture of FGA and 7-ADCA to
for example a pH between 8.0 and 9.0 and then
acidifying the mixture again to a pH between 6.5 and
20   8.5.

Another example of the process according to
the invention is its application in the enzymatic
preparation of amoxicillin from 6-APA and D-p-
hydroxyphenyl glycine methyl ester (FGHM) and in the
25   preparation of cefadroxil from 7-ADCA and FGHM. The
solubility of FGHM is relatively low and decreases with
increasing pH, whereas the solubility of 6-APA and 7-
ADCA is also relatively low and decreases with
decreasing pH. It has been found that subjecting a
30   mixture of for example 6-APA or 7-ADCA and FGHM to a pH
decrease to a value at which FGHM is virtually
completely dissolved (for example a pH value between 5
and 6) and next to a pH increase to a value between for

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example 6 and 7.5 enables a supersaturation of FGHM by
a factor of 3-5 to be achieved. Thus, the acylation
reaction can be carried out at a somewhat higher pH,
with a higher concentration of dissolved β-lactam
5   nucleus, whilst the concentration of the acylation
agent is also relatively high.

Another embodiment is to dissolve 7-ADCA to
a relatively high concentration with a base to a pH
between for example 8 and 9 and then adding a
10 concentrated, acid solution of FGHM, in which process
the pH decreases. In this embodiment, supersaturation
of both 7-ADCA and FGHM can be achieved at the same
time. The enzymatic reaction can be started after all
reactants have been added. It is also possible to add
15 (a portion of the) concentrated FGHM or 7-ADCA solution
(or slurry) during the reaction.

Another example of the process according to
the invention is its application in the enzymatic
preparation of cefazolin from tetrazole-1-acetic acid
20 and as a β-lactam nucleus 7-aminocephalosporanic acid
(7-ACA) or 7-amino-3-(5-methyl-1,3,4-thiadiazole-2-yl-
thiomethyl)-3-cef-em-4-carboxylic acid (7-ACA-MMTD). As
the acylating agent does not contain an α-amino group,
a so-called thermodynamically controlled coupling
25 reaction with the acid may be performed. The optimum pH
for such thermodynamically controlled coupling reaction
is preferably between 4.0 and 6.5. At this pH the
solubility of the β-lactam nucleus is relatively low.
It has appeared that the conversion in the coupling
30 reaction can be strongly enlarged by adding a
concentrated solution of the β-lactam nucleus with a
relatively high pH (for instance between 7.0 and 9.0)

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to the enzymatic reaction mixture, while the pH of the
reaction mixture is kept at a value between 4.0 and
6.5, e.g. by titration with an acid.

5   The process according to the invention can
suitably be applied in the preparation of β-lactam
antibiotics, for example cephalexin, ampicillin,
cefaclor, amoxicillin, cephradine, cefadroxil,
cefotaxime, cefazolin, cefprozil, loracarbef and
cefaloglycin.

10   Any β-lactam nucleus can in principle be
used, in particular a β-lactam nucleus with the general
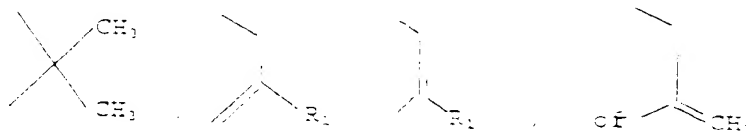formula (1)

15

20
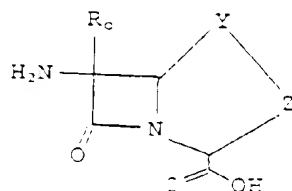
where R_0 represents H or an alkoxy group having 1-3 C
atoms; Y represents CH_2, O, S or an oxidized form of
25 sulphur; and Z represents

30

where R_1 represents for example H, OH, halogen, an
alkoxy group having 1-5 C atoms, an alkyl group having
35 1-5 C atoms, a cycloalkyl group having 4-8 C atoms, an

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aryl or a heteroaryl group having 6-10 C atoms, in
which the groups may or may not be substituted with for
example an alkyl, an aryl, a carboxy or an alkoxy group
having 1-8 C atoms; and where the carboxylic acid group
5   may be an ester group if so desired.

Suitable examples of β-lactam nuclei that
may be employed in the process according to the
invention are penicillin derivatives, for example 6-
aminopenicillanic acid (6-APA) and cephalosporic acid
10 derivatives, for example a 7-aminocephalosporanic acid
with or without a substituent at the 3-site, for
example 7-aminocephalosporanic acid (7-ACA), 7-
aminodesacetoxycephalosporanic acid (7-ADCA), 7-amino-
3-chloro-cef-3-em-4-carboxylic acid (7-ACCA), 7-amino-
15 3-(1-propenyl)-cef-3-em-4-carboxylic acid (7-PACA), 7-
amino-3-(5-methyl-1,3,4-thiadiazole-2-yl-
thiomethyl)cef-3-em-4-carboxylic acid (7-ACA-MMTD)
and 7-amino-3-chloro-8-oxo-1-azabicyclo[4.2.0]oct-2-
ene-2-carboxylic acid.
20   In the (enzymatic) acylation reaction, the
acylation agent may be for instance a phenyl glycine in
activated form, preferably a (primary, secondary or
tertiary) amide or salt thereof, or a lower alkyl (1-
4C) ester, for instance a methyl ester; suitable phenyl
25 glycines are for example substituted and unsubstituted
phenyl glycines, in particular phenyl glycine, p-
hydroxyphenyl glycine, dihydrophenyl glycine. In
addition α-substituted acetic acid derivatives and the
corresponding amides and esters may be applied, for
30 instance phenyl acetic acid, phenoxy acetic acid,
tetrazole-1-acetic acid, mandelic acid or thienyl
acetic acid.

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In principle, any enzyme that is suitable as a catalyst in the coupling reaction can be used as the enzyme. Such enzymes include the enzymes collectively referred to as penicillin amidase or
5 penicillin acylase. Such enzymes are described in for example J.G. Shewale et al., Process Biochemistry, August 1989, pp. 146-154 and in J.G. Shewale et all, Process Biochemistry International, June 1990, pp. 97-103. Examples of suitable enzymes are enzymes derived
10 from Acetobacter, in particular Acetobacter pasteurianum, Aeromonas, Alcaligenes, in particular Alcaligenes faecalis, Aphanocladium, Bacillus sp., in particular Bacillus megaterium, Cephalosporium, Escherichia, in particular Escherichia coli,
15 Flavobacterium, Fusarium, in particular Fusarium oxysporum and Fusarium Solani, Kluyvera, Mycoplana, Protaminobacter, Proteus, in particular Proteus rettgari, Pseudomonas and Xanthomonas, in particular Xanthomonas citrii.
20 Preferably an immobilized enzyme is used, since in that case the enzyme can be easily isolated and re-used.

Particularly suitable enzymes among the immobilized enzymes that are commercially available are
25 for example the Escherichia coli enzyme from Boehringer Mannheim GmbH, which is commercially available under the name Enzygel[R], the immobilized Penicillin-G acylase from Recordati and the immobilized Penicillin-G acylase from Pharma Biotechnology Hannover. In addition,
30 enzymes may also be utilized in crystalline form (CLEC's[TM]).

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The temperature at which the enzymatic acylation reaction is effected usually is below 40°C, preferably between -5 and 35°C. The pH at which the enzymatic acylation reaction is effected usually is
5   between 3.0 and 9.5, preferably between 4.0 and 9.0. The optimum pH for a kinetically controlled coupling reaction is relatively high, for instance between 4.5 and 9.0, preferably between 5.5 and 8.5, in particular between 6.0 and 8.0. The optimum pH of a
10   thermodynamically controlled coupling reaction generally is lower and lies for instance between 3.0 and 7.0, preferably between 4.0 and 6.5.

The reaction preferably is stopped almost completely when maximum conversion has virtually been
15   achieved. A suitable embodiment for stopping the reaction is to lower the pH, preferably to a value between 4.0 and 6.3, in particular between 4.5 and 5.7. Another suitable embodiment is to lower the temperature of the reaction mixture on attaining the maximum
20   conversion. A combination of the two embodiments is possible also.

Once the reaction has been stopped on attaining the maximum conversion, the reaction mixture usually is present in the form of a suspension
25   comprising a plurality of solids, for example the antibiotic, D-phenyl glycine and, possibly, immobilized enzyme. The immobilized enzyme preferably is recovered in the interest of process economics. This can suitably be accomplished by for example by filtering the
30   reaction mixture on a sieve, while stirring, the stirrer's direction of rotation being chosen so that the suspension is pumped upwards at the centre of the

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stirrer. Subsequently, valuable components such as the
antibiotic and FG can be recovered by means of for
example a pH change.

        For the purposes of the invention, a pH
5   change can be brought about by adding an acid. Suitable
acids are for example mineral acids, in particular
sulphuric acid, hydrochloric acid or nitric acid and
carboxylic acids, for example acetic acid, oxalic acid
and citric acid. A pH increase can be brought about by
10   for example adding a base. Suitable bases are for
example inorganic bases, in particular ammonium
hydroxide, potassium hydroxide or sodium hydroxide, and
organic bases, for example triethyl amine and FGA.
Preferably, ammonium hydroxide is used.
15   The enzymatic acylation reaction and the
measures mentioned, for example the preparation of the
supersaturated mixtures, can be effected in water. If
desired, the reaction mixture may also contain an
organic solvent or a mixture of organic solvents,
20   preferably less than 30 vol.%. Examples of suitable
organic solvents are alcohols having 1-7 C atoms, for
example a monoalcohol, in particular methanol or
ethanol; a diol, in particular ethylene glycol, or a
triol, in particular glycerol.
25   The molar ratio of acylation agent to β-
lactam nucleus, i.e. the total amount of acylation
agent added, divided by the total amount of β-lactam
nucleus added, expressed in moles, is less than 2.5. It
is preferred for the molar ratio to be between 0.5 and
30   2.0, in particular between 0.7 and 1.8.
        The enzymatic acylation reaction is
preferably carried out as a batch process. If desired,

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the reaction can also be carried out continuously.

The invention will be further elucidated by means of the following examples, without however being restricted thereto.

5

Abbreviations:

7-ACCA: 7-amino-3-chloro-cef-3-em-4-carboxylic acid

7-ADCA: 7-aminodesacetoxycephalosporanic acid

6-APA: 6-amino-penicillanic acid

10 AMPI: ampicillin

CCl: cefaclor

CEX: cephalexin

FG: D-phenyl glycine

FGA: D-phenyl glycine amide

15 FGH: D-p-hydroxyphenyl glycine

FGHM: D-p-hydroxyphenyl glycine methyl ester

Assemblase™ is an immobilized Escherichia coli penicillin acylase from E. coli ATCC 1105 as
20 described in WO-A-97/04086. The immobilization is effected as set out in EP-A-222462, with gelatin and chitosan being used as gelating agents and glutaraldehyde as crosslinking agent.

The ultimate activity of the Escherichia
25 coli penicillin acylase is determined by the amount of enzyme added to the activated globules and amounted to 3 ASU/g of dry weight. 1 ASU (Amoxicillin Synthesis Unit) being defined as the amount of enzyme capable of producing 1 g of Amoxicillin.$3H_2O$ from 6-APA and FGHM
30 per hour (at 20°C; 6.5% 6-APA and 6.5% FGHM).

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Comparative Example 1

Synthesis of cefaclor (7-ACCA not supersaturated)

An enzyme reactor (1.5 l, diameter 11 cm),
fitted with a 175 μm mesh sieve bottom, was filled with
5   100 g of net-wet Assemblase™.

A preparation reactor (1.2 l) was filled
with 75.4 g of FGA (0.500 mole), 4.0 g of sodium
bisulphite, 700 g of water, 6.6 g of 4N H_2SO_4 and 72.1 g
of 7-ACCA (0.300 mol). This mixture was stirred at T =
10   10°C; the pH was 7.5. Subsequently, the mixture was
transferred into the enzyme reactor with the aid of 118
ml of water (T = 10°C).

The stirrer in the enzyme reactor was
switched on at t = 0. The temperature was kept at 10°C.
15   The pH was kept at 7.5 by titration with 4N H_2SO_4.
At t = 48 minutes the enzyme reactor contained approx.:

215 mmol CCl (conversion = 72%)

70 mmol 7-ACCA

175 mmol FGA

20   95 mmol FG

At t = 81 minutes the enzyme reactor contained approx.:

212 mmol CCl (conversion = 71%)

61 mmol 7-ACCA

83 mmol FGA

25   182 mmol FG

Thereafter, the amount of CCl decreased. The
concentrations (C) in mmol/kg of FGA in solution (FGA_s),
total FGA (FGA_t), 7-ACCA in solution ($ACCA_s$), total 7-
ACCA ($ACCA_t$) and total CCL (CCL_t), during the reaction
30   are shown in Fig. 1 as a function of time (T) in
minutes (min.).

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Example I

Synthesis of Cefaclor (7-ACCA supersaturated)

An enzyme reactor (1.5 l, diameter 11 cm),
fitted with a 175 μm mesh sieve bottom, was filled with
5   300 g of net-wet Assemblase ™.

A preparation reactor (1.2 l) was filled
with 86.6 g of 7-ACCA (0.360 mol), 67.8 g of FGA (0.450
mol), 4.0 g of sodium bisulphite and 402 g of water.
This mixture was stirred for 5 minutes at T = 10°C; the
10   pH was 7.4.

The pH was brought to 8.0, while stirring,
with the aid of 16.8 g of concentrated ammonium
hydroxide. Subsequently, the pH was lowered from 8.0 to
6.4 by metering in 73.8 ml of 4N H_2SO_4 in 20 minutes.
15   Next, at t = 0, the mixture was transferred from the
preparation reactor to the enzyme reactor with the aid
of 140 ml of water (T = 10°C).

The stirrer in the enzyme reactor was
switched on at t = 0; T = 10°C. The pH was kept at 6.4
20   by titration with 4N H_2SO_4. After 7 hours, 66.0 ml of
acid had been added. The reactor now contained:

300 mmol CCl (conversion = 83%)

55 mmol 7-ACCA

100 mmol FGA

25   45 mmol FG

The concentrations (C) in mmol/kg of FGA in solution
(FGA_s), total FGA (FGA_t), 7-ACCA in solution ($ACCA_s$),
total 7-ACCA ($ACCA_t$) and total CCL (CCL_t), during the
reaction are shown in Fig. I as a function of time (T)
30   in minutes (min.).

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Example II

Synthesis of Cefaclor (7-ACCA supersaturated and 7-ACCA
being metered in during the enzymatic reaction)

An enzyme reactor (1.5 l, diameter 11 cm),
5   fitted with a 175 μm mesh sieve bottom, was filled with
150 g of net-wet Assemblase™.

A preparation reactor (1.2 l) was filled
with 48.1 g of 7-ACCA (0.200 mol), 75.4 g of FGA (0.500
mol), 4.0 g of sodium bisulphite and 175 g of water.
10   This mixture was stirred for 5 minutes at T = 10°C; the
pH was 7.43.

The pH was brought to 9.0, while stirring,
with the aid of 15.5 g of concentrated ammonium
hydroxide. Subsequently, the pH was lowered from 9.0 to
15   6.4 by metering in 80.6 ml of 6N H_2SO_4 in 45 minutes.
Next, at t = 0, the mixture was transferred from the
preparation reactor to the enzyme reactor with the aid
of 40 g of water (T = 10°C).

The stirrer in the enzyme reactor was
20   switched on at t = 0. The temperature was kept at T =
10°C. 244 g (0.200 mol) of 7-ACCA solution were metered
in at a constant rate in 109 minutes. The solution had
been freshly prepared by suspending 48.1 g of 7-ACCA
(0.200 mol) in 183 g of water at T = 3°C and raising the
25   pH to 8.2 with the aid of 13.2 concentrated NH_3, in
which process all 7-ACCA dissolved. From t = 0 onwards,
the pH in the enzyme reactor was kept at 6.4 by
titration with 6N H_2SO_4. At t = 350 minutes the enzyme
reactor contained:
30   350 mmol CCl (conversion = 88%)
                45 mmol 7-ACCA
                80 mmol FGA

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65 mmol FG

At t = 400 minutes the amount of Cefaclor was maximum
and the pH was lowered to 5.0 by adding 6N H_2SO_4. The
5 enzyme reactor now contained:

   370 mmol CCl (conversion = 92%)

   25 mmol 7-ACCA

   40 mmol FGA

   85 mmol FG

10 The concentrations (C) in mmol/kg of FGA in solution
(FGA_s), total FGA (FGA_t), 7-ACCA in solution ($ACCA_s$),
total 7-ACCA ($ACCA_t$) and total CCL (CCL_t), during the
reaction are shown in Fig. 3 as a function of time (T)
in minutes (min.).

15

Comparative Experiment B

Synthesis of Ampicillin (6-APA not supersaturated)

   First a solution of FGA.1/2 H_2SO_4 was
prepared. 301.6 g of FGA (2.00 mol) were suspended in
20 650 g of water at T = 5°C. 102.1 g of 96% H_2SO_4 (1.00
mol) were added dropwise while stirring, with the
temperature being kept at T < 25°C by means of cooling.

   Next the enzymatic condensation was
performed. An enzyme reactor (1.5 l, diameter 11 cm),
25 fitted with a 175 μm mesh sieve bottom, was filled with
300 g of net-wet Assemblase™.

   A preparation reactor (1.2 l) was filled
with 131.6 g of 6-APA (0.600 mol), 30.2 g of FGA (0.200
mol) and 400 ml of water (T = 10°C). This mixture was
30 stirred for 15 minutes at T = 10°C and subsequently, at
t = 0 transferred into the enzyme reactor with the aid
of 100 ml of water (T = 10°C).

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The stirrer in the enzyme reactor was switched on at t = 0. 423.7 g (0.800 mol) of FGA.1/2H$_2$SO$_4$ were added to the solution at a constant rate in 283 minutes, with the temperature being kept at
5   10°C. The pH was 6.3. From t = 328 minutes onwards, the pH was kept at 6.3 by titration with 6N H$_2$SO$_4$.

At t = 540 minutes the amount of Ampicillin was maximum and the pH was lowered to 5.6 by adding 6N H$_2$SO$_4$.

10   The enzyme reactor now contained:

575 mmol AMPI (=96% relative to the amount
of 6-APA   employed)

15 mmol 6-APA

50 mmol FGA

15   365 mmol FG

The concentrations (C) in mmol/kg of FGA in solution (FGA$_s$), total FGA (FGA$_t$), 6-APA in solution (APA$_s$), total 6-APA (APA$_t$) and total Ampi (Ampi$_t$) occurring during the reaction are shown in Fig. 4 as a function
20   of time (T) in minutes (min.).

Example III

Synthesis of Ampicillin (6-APA supersaturated)

An enzyme reactor (1.5 l. diameter 11 cm),
25   fitted with a 175 μm mesh sieve bottom. was filled with 300 g of net-wet Assemblase™.

A preparation reactor (1.0 l) was filled with 550 ml of water (T = 10°C), 138.8 g (0.920 mol) of FGA and 131.6 g (0.600 mol) of 6-APA. After 5 minutes
30   at 10°C a clear solution obtained with a pH value of 7.4. Subsequently, the pH was brought to 6.5 with 96% H$_2$SO$_4$ (ca. 16 g) and after stirring for 5 minutes at

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10°C the solution was transferred to the enzyme reactor
at t = 0 with the aid 100 ml of water (T = 10°C).

At t = 0 the temperature was 10°C and the pH
= 6.3. During the enzyme reaction the pH was kept at
5   6.3 by titration with 6N H_2SO_4. At t = 300 minutes the
amount of AMPI was maximum and the pH was lowered to
5.6 by adding 6N H_2SO_4.
The enzyme reactor now contained:

575 mmol AMPI (= 96% relative to the amount
10   of 6 APA employed)
15 mmol   of 6-APA
40 mmol   of FGA
295 mmol FG

The concentrations (C) in mmol/kg of FGA in solution
15   (FGA_s), total FGA (FGA_t), 6-APA in solution (APA_s),
total 6-APA (APA_t) and total Ampi ($Ampi_t$), occurring
during the reaction are shown in Fig. 5 as a function
of time (T) in minutes (min.).

20 Comparative Example C
Synthesis of Cephalexin (7-ADCA not supersaturated)

An enzyme reactor (1.5 l, diameter 11 cm),
fitted with a 175 μm mesh sieve bottom, was filled with
75 g of net-wet Assemblase™.
25   A preparation reactor (1.2 l) was filled
with 668 g of water (T = 4°C), 4.0 g of sodium
bisulphite, 130.3 g of 7-ADCA (0.600 mol) and 75.4 g of
FGA (0.500 mol). 7.8 g of concentrated ammonium
hydroxide were added, whereupon the suspension was
30 stirred for 15 minutes at T=4°C. The pH was 7.8.

Subsequently, at t=0, the suspension was
transferred into the enzyme reactor with the aid of 50

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ml of water (T = 4°C). The stirrer in the enzyme reactor
was switched on at t = 0. The temperature was kept at
T=4°C all the time. All 7-ADCA had dissolved after
approximately 75 minutes; thereafter, a clear solution,

5   apart from solid Assemblase™, was present. After 210
minutes the pH had risen to 8.6.
The reactor now contained:

                393 mmol CEX (conversion = 66%; S/H = 6.1)
                200 mmol 7-ADCA
10   64 mmol FG
                33 mmol FGA.

S/H (in mmol CEX/mmol FG) as a function of conversion
(in %) is shown in Fig. 6.

15   Example IV
Synthesis of Cephalexin (7-ADCA supersaturated)
                An enzyme reactor (1.5 l, diameter 11 cm),
fitted with a 175 μm mesh sieve bottom, was filled with
75 g of net-wet Assemblase™.

20   A preparation reactor (1.2 l) was filled
with 600 ml of water (T = 4°C), 4.0 g of sodium
bisulphite, 130.3 g of 7-ADCA (0.600 mol) and 90.5 g of
FGA (0.600 mol). The suspension was stirred for 15
minutes at T=4°C. The pH was 7.6.

25   The pH was brought to 8.6, with stirring,
using 43.8 g of concentrated ammonium hydroxide.
Stirring was effected for 5 minutes at T=4°C.
Subsequently, 21.2 g of concentrated H_2SO_4 were added.
The clear solution was stirred for 15 minutes at T=4°C.
30   The pH was 7.4.

                Subsequently, at t=0, the suspension was
transferred into the enzyme reactor with the aid of 50

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ml of water (T = 4°C). The stirrer in the enzyme reactor
was switched on at t = 0. The temperature was kept at
T=4°C all the time. At t=90 minutes the pH had risen to
8.05. 5.5 g of concentrated H_2SO_4 were added, causing
5   the pH to decrease to 7.75. A clear solution, apart
from solid Assemblase™, was present throughout the
enzyme reaction.
After 340 minutes the pH had risen to 8.5.
The reactor now contained:
10   445 mmol CEX (conversion = 74%; S/H - 7.2)
                    146 mmol 7-ADCA
                    62 mmol FG
                    80 mmol FGA.
S/H (in mmol CEX/mmol FG) as a function of conversion
15   (in %) is shown in Fig. 6.

Example V
Synthesis of Cephalexin (7-ADCA supersaturated)
                    An enzyme reactor (1.5 l, diameter 11 cm),
20   fitted with a 175 μm mesh sieve bottom, was filled with
75 g of net-wet Assemblase™.
                    A preparation reactor (1.2 l) was filled
with 500 ml of water (T = 4°C), 4.0 g of sodium
bisulphite. 162.9 g of 7-ADCA (0.750 mol) and 113.1 g
25   of FGA (0.750 mol). The suspension was stirred for 15
minutes at T=4°C. The pH was 7.6.
                    The pH was brought to 8.7, with stirring,
using 55.6 g of concentrated ammonium hydroxide.
Stirring was effected for 5 minutes at T=4°C.
30   Subsequently, 9.6 g of concentrated H_2SO_4 were added.
The clear solution was stirred for 15 minutes at T=4°C.
The pH was 8.0.

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Subsequently, at t=0, the suspension was
transferred into the enzyme reactor with the aid of 50
ml of water (T = 4°C). The stirrer in the enzyme reactor
was switched on at t = 0. The temperature was kept at
5   T=4°C all the time. After 60 minutes the pH had risen to
8.3. The pH was now kept at 8.3 by titration with
concentrated H_2SO_4.
At t=300 minutes a total of 23.4 g of concentrated H_2SO_4
had been titrated. At this point titration was stopped;
10 at t=450 minutes the pH had risen to 8.7.
A clear solution, apart from solid
Assemblase™, was present throughout the enzyme
reaction.
At t=450 minutes the reactor contained:
15   545 mmol CEX (conversion = 73%; S/H = 8.5)
            195 mmol 7-ADCA
            64 mmol FG
            129 mmol FGA.
S/H (in mmol CEX/mmol FG) as a function of conversion
20 (in %) is shown in Fig. 6.

Comparative Experiment D
Synthesis of Cefadroxil (neither 7-ADCA nor FGHM
supersaturated)
25   An enzyme reactor (1.5 l, diameter 11 cm),
fitted with a 175 μm mesh sieve bottom, was filled with
300 g of net-wet Assemblase™.
A preparation reactor (1.2 l) was filled
with 63.7 ml of 7-ADCA (0.293 mol), 107.1 g of FGHM
30 (0.590 mol), 4.0 g of sodium bisulphite and 440 g of
water. This mixture was stirred for 5 minutes at T=10°C
with the pH being kept at 7.0 with the aid of 7.5 g of

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concentrated ammonium hydroxide.

At t=0, the suspension was transferred from the preparation reactor into the enzyme reactor with the aid of 50 ml of water (T = 10°C). The stirrer in the
5   enzyme reactor was switched on at t = 0. The temperature was kept at T=10°C; the pH remained constant at 7.0 (no titration).

After 380 minutes the enzyme reactor contained:

	68 mmol	Cefadroxil (conversion = 23%;
10		S/H = 0.67)
	222 mmol	7-ADCA
	420 mmol	FGHM
	101 mmol	FGH

The concentrations (C) in mmol/kg of total Cefadroxil
15   (Cefadroxil$_t$), total FGH (FGH$_t$), total FGHM (FGHM$_t$), FGHM in solution (FGHM$_s$), total 7-ADCA (7-ADCA$_t$) and 7-ADCA in solution (7-ADCA$_s$), occurring during the reaction are shown in Fig. 7, as a function of time (T) in minutes (min.).

20

Example VI

Synthesis of Cefadroxil (both FGHM and 7-ADCA supersaturated)

An enzyme reactor (1.5 l, diameter 11 cm),
25   fitted with a 175 µm mesh sieve bottom, was filled with 270 g of net-wet Assemblase™.

A preparation reactor (1.2 l) was filled with 97.7 ml of 7-ADCA (0.448 mol), 4.0 g of sodium bisulphite and 250 g of water (T=10°C). The suspension
30   was stirred for 5 minutes at T=10°C. Subsequently, the pH was brought to 8.1 with the aid of 40.3 g of NH$_3$, in which process a clear solution evolved (T=10°C).

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A second preparation reactor was filled
with 98.5 g of FGHM (0.538 mol), 60 g of water (T=10°C)
and 105.1 g of 6N H_2SO_4 solution. This solution was
brought to T=10°C; pH = 2.3.

5   The 7-ADCA solution from the first
preparation reactor was transferred to the enzyme
reactor with the aid of 20 ml of water (T=10°C). The
stirrer in the enzyme reactor was switched on. From t=0
the FGHM solution from the second preparation reactor

10   was metered into the enzyme reactor at a constant rate
in 120 minutes. The temperature was kept at T=10°C.
After 30 minutes the pH had decreased from
8.1 to 6.8. Next, the pH was maintained at 6.8 by
titration with a concentrated ammonium hydroxide.

15   At t=120 minutes, 8.3 g of concentrated ammonium
hydroxide had been added. Titration was stopped; the pH
continued to be 6.8.
After 420 minutes the enzyme reactor contained:
        390 mmol Cefadroxil (conversion = 87%;

20   S/H = 4.0)
        50 mmol   7-ADCA
        44 mmol   FGHM
        97 mmol   FGH
The concentrations (C) in mmol/kg of total Cefadroxil

25   (Cefadroxil$_t$), total FGH (FGH$_t$), total FGHM (FGHM$_t$),
FGHM in solution (FGHM$_s$), total 7-ADCA (7-ADCA$_t$) and 7-
ADCA in solution (7-ADCA$_s$), occurring during the
reaction are shown in Fig. 8, as a function of time (T)
in minutes (min.).

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CLAIMS

1.   Process for the preparation of a β-lactam
antibiotic in which a β-lactam nucleus is
subjected to an enzymatic acylation reaction with
the aid of an acylation agent at a molar ratio of
acylation agent/β-lactam nucleus of less than
2.5, characterized in that the acylation agent
and/or the β-lactam nucleus are/is supersaturated
in the reaction mixture during at least part of
the acylation reaction.

2.   Process according to Claim 1, in which the
acylation agent and/or the β-lactam nucleus
are/is supersaturated in the reaction mixture at
the beginning of the acylation reaction.

3.   Process according to Claim 1 or Claim 2, in which
a concentrated slurry or solution of the β-lactam
nucleus and/or the acylation agent with a
different pH or a higher temperature than the pH
or temperature at which the acylation reaction is
carried out is added to the reaction mixture
during the acylation reaction.

4.   Process according to any one of Claims 1-3 in
which the β-lactam nucleus is supersaturated in
the reaction mixture.

5.   Process according to Claim 4 in which a mixture
in which the β-lactam nucleus is dissolved is
subjected to a pH decrease or a pH increase until
a pH between 3.0 and 9.0 is reached.

6.   Process according to Claim 5 in which the pH
decrease or pH increase is effected until a pH
between 4.0 and 8.5 is reached.

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7. Process according to any one of Claims 1-6 in which the acylation agent is supersaturated in the reaction mixture.

8. Process according to Claim 7 in which a mixture in which the acylation agent is dissolved is subjected to a pH increase.

9. Process according to Claim 8 in which the pH is increased to a pH higher than 5.5.

10. Process according to Claim 9 in which the pH is increased to a pH higher than 6.

11. Process according to any one of Claims 4-10 in which a mixture containing dissolved β-lactam nucleus and/or acylation agent is subjected to a temperature decrease.

12. Process according to Claim 11 in which the temperature is decreased to a temperature below 15°C.

13. Process according to Claim 12 in which the temperature is decreased to a temperature below 10°C.

14. Process according to any one of Claims 7-13 in which the methyl ester of p-hydroxyphenyl glycine is used as acylation agent.

15. Process according to any one of Claims 1-13 in which an amide is used as acylation agent.

16. Process according to any one of Claims 1-15 in which 7-aminodesacetoxycephalosporanic acid (7-ADCA), 7-amino-3-chloro-cef-3-em-4-carboxylic acid (7-ACCA), 6-aminopenicillanic acid (6-APA), 7-aminocefalosporanic acid (7-ACA), 7-amino-3-(1-propenyl)-cef-3-em-4-carboxylic acid (7-PACA), 7-amino-3-(5-methyl-1,3,4-thiadiazole-2-yl-

thiomethyl)-cef-3-em-4-carboxylic acid (7-ACA-
MMTD) or 7-amino-3-chloro-8-oxo-1-
azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid is
used as β-lactam nucleus.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12P35/04   C12P37/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12P   C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 02663 A (GIST BROCADES BV ;CLAUSEN KIM (DK); DEKKERS ROCUS MARINUS (NL)) 1 February 1996 see claims	1-16

X	WO 92 01061 A (NOVONORDISK AS) 23 January 1992 cited in the application see claims	1-16

A	WO 97 22610 A (CHEMFERM VOF ;BOESTEN WILHELMUS H J (NL); DEN TWEEL WILHELMUS JOHA) 26 June 1997 see claims	1-16

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search	Date of mailing of the international search report
22 December 1998	04/01/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl Fax: (+31-70) 340-3016	Chouly. J

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Inter......onal Application No

PCT/NL 98/00570

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 23897 A (CHEMFERM VOF ;BOESTEN WILHELMUS H J (NL); DOOREN THEODORUS JOHANNE) 8 August 1996 cited in the application see claims	1-16

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